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Insulin and leptin receptors as possible new candidates for endocrine control in normal and disturbed human pregnancy

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Abstract:

Leptin and Insulin are secreted into the maternal and to a lesser extent into the fetal blood stream where they act as placental signals and nourish the fetus, making them possible candidates for the endocrine control of the placenta. We investigated differences in Leptin (LR) and Insulin receptor (IR) expression in normal and disturbed first trimester human pregnancy at protein level by immunohistochemistry and at mRNA level by real time RT-PCR (TaqMan).

Highest expression of LR and IR was present in villous (VT) and extravillous trophoblasts (EVT). In hydatidiform mole trophoblasts, significantly higher LR and IR expression was observed as compared to normal pregnancy. Additionally, LR and IR were also expressed in glandular epithelial cells of the decidua, again to the highest extent in hydatidiform mole as compared to normal pregnancy.

With regard to abortive placentas, significant differences were also present as compared to normal first trimester placenta in the expression of LR and IR in VT, EVT and in glandular epithelial cells of the decidua. Results at protein expression of LR and IR were confirmed at mRNA level.

The majority of IR and LR are expressed on structures that are currently assumed to drive placental growth. LR and IR are strongly up-regulated in placentas of hydatidiform mole and abortion. Our findings may suggest IR and LR as possible new candidates for the endocrine control of human pregnancy.

Keywords: abortion, first trimester pregnancy, hydatidiform mole, insulin receptor, leptin receptor

Introduction

Immunologic, endocrine, metabolic and vascular regulatory mechanisms being genetically controlled, are involved in the success of human pregnancy. Disturbances of any of these regulatory processes can lead to fetal loss. However, 25-50% of reproductive-aged women experience one or more miscarriages, often due to fetal chromosomal abnormalities, especially with increasing maternal age (Toth *et al*., 2007a). Furthermore, 1–3% of women during child-bearing years suffer from recurrent miscarriage (RM), the occurrence of three or more consecutive spontaneous miscarriages regardless of previous live births (Regan *et al*., 2000). In nearly 50% of affected patients, the cause of RM remains unknown leading to investigations on new risk factors and possible treatment strategies (Regan *et al*., 2000). Along these new risk factors leptin and insulin as well as their receptors seem to play a possible role in the maintenance of normal pregnancy (Toth *et al*., 2008).

Leptin was originally identified as an adipocyte-derived protein and is a regulator of satiety and energy homeostasis. Nearly 50% of circulating leptin is bound to plasma proteins contributing to steady-state plasma leptin levels (Toth et al., 2007b). Furthermore, leptin seems to play a functional role in the implantation process as it stimulates matrix metalloproteinase (MMP) expression in cytotrophoblasts (Sagawa *et al*., 2002a) and in fetal developments, as its levels in the umbilical vessels have been related with birth weight (Papageorgiou et al., 2004; Harigaya et al., 1997). It also modulates glucose metabolism by increasing insulin sensitivity (Sagawa *et al*., 2002b). Yamashita *et al*. (2001) were able to demonstrate that animals with mutations in the leptin receptor (LR) gene develop gestational diabetes during pregnancy. In human pregnancy, leptin-receptor gene expression

was the only angiogenesis-related gene which was up-regulated in chorionic villi of RM patients as compared to normal pregnancy (Choi *et al.,* 2003).

So far, little is known on the expression of the insulin receptor (IR) in disturbed pregnancies. However, there is evidence that the IR regulates choriocarcinoma cell invasion (Diaz *et al.,* 2007). In human placenta of normal pregnancy, the IR distribution pattern is characterized by a spatiotemporal change between first trimester and term (Desoye *et al.,* 2007). While the IR is found predominantly on the maternal side (syncytiotrophoblast, cytotrophoblast) in first trimester pregnancy, it is expressed on the fetal side (fetal vessels) at term (Desoye *et al.,* 2007).

We were able to show leptin expression in normal and disturbed pregnancy (Toth *et al.,* 2008). Leptin expression was lowest in placentas of miscarriage patients and highest in placentas of mole pregnancies, whereas expression of leptin in glandular epithelial cells of the decidua was increased in miscarriage as compared to normal pregnancy. Leptin expressing cells at the feto-maternal interface were identified as extravillous trophoblast (EVT) by double immunofluorescence and Cytokeratin 7 (CK7) staining.

The present study was designed to improve our understanding of the role of LR and IR in placental and fetal development. The frequency and tissue distribution patterns of LR and IR in the first trimester of normal human pregnancy, abortion and hydatidiform mole were investigated.

Material and methods

Samples of paraffin-wax embedded placental tissue were randomly obtained from women with spontaneous abortion (n=14), hydatidiform mole (n=14) or termination of normal pregnancy (n=14), each from the 6^{th} to 12^{th} week of pregnancy. Demographic and clinical data of the study population are summarized in table 1. The exact number of slides of each week of gestational age is shown in table 2.

Signed informed consent was obtained from all participants allowing analysis of all clinical and laboratory data mentioned in this paper. The Human Investigation Review Board of the Ludwig-Maximilians- University Munich approved the study.

Tissue storage was performed as followed: placentas were put on ice immediately after curettage and placental tissue pieces for RNA isolation were stored in RNAlater© buffer solution (Ambion, Darmstadt, Germany). Within 30 min RNA isolation from placental tissue was started.

Immunohistochemistry

For immunohistochemistry, paraffin sections were deparaffinised in xylol, incubated with Methanol/H_2O_2 (20 min) to inhibit endogenous peroxidase activity, rehydrated in alcohol gradient to PBS and subsequently incubated with mouse serum for LR and rabbit serum for IR antibody (20 min, 22 °C) Then the slides were incubated with the primary antibodies (table 3), the LR antibody for one hour at room temperature, the IR antibody overnight at 4 °C. The Vectastain® Elite ABC-Kit (Vector Laboratories, Peterborough, UK) was used for visualization according to manufacturer instructions. Finally, slides were counterstained with hemalaun (2 min) and then cover-slipped.

As positive control tissue for LR and IR, placental slides of normal and term pregnancy were used. Additionally, slides of human pancreas were used for IR. For negative controls, primary antibodies were replaced by horse serum.

The intensity and distribution patterns of antigen expression were evaluated by using a semi quantitative method (immunoreactive score (IRS) as previously described (Remmele *et al*., 1987). Briefly, the IRS score was calculated by multiplication of optical staining intensity (graded as 0=none, 1=weak, 2=moderate and 3=strong staining) and the percentage of positive staining cells (0=no staining, 1 ≤10% of the cells, 2=11-50% of the cells, 3=51-80% of the cells and 4 ≥81% of the cells). Sections were examined using a Leitz (Wetzlar, Germany) photomicroscope. Digital images were obtained with a digital camera system (JVC, Japan) and were stored on computer.

The SPSS/PC software package version 16.0 was used for collection, processing, and statistical data analysis. Statistical analysis was performed using the non-parametrical Mann-Whitney-U signed rank test for comparison of the means, $p<0.05$ values were considered statistically significant.

RNA extraction from placental tissue

A total amount of 5 x 10 mg abortive and 5 x 10 mg normal control placental tissue ranging from the 7^{th} to 12^{th} week of gestation was used for extraction of mRNA. Total RNA was investigated by NucleoSpin® RNAII Kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's protocol. Purified RNA was quantified and evaluated for purity by UV spectrometry.

Reverse transcription

Reverse Transcription (RT) was carried out with the “High Capacity cDNA Reverse Transcription Kit” (Applied Biosystems, Weiterstadt, Germany) according to the protocol in a mastercycler gradient (Eppendorf, Hamburg, Germany). RT conditions were: 10 min 25°C, 2h 37°C, 5 sec 85°C and 4°C on hold.

Real-Time RT-PCR

Real-time RT-PCR reactions were performed in quadruplicate in optical 96-well reaction microtiter plates covered with optical caps, in a volume of 20 µl containing 1 µl TaqMan® Gene Expression Assay 20x (Hs00174492_m1 for LR Exon boundary 19-20, Hs00174497_m1 for LR Exon boundary 6-27, Hs00213886_m1 for Leptin receptor overlapping transcript and Hs00415243_m1 for Leptin receptor overlapping transcript-like1 mRNA detection and Hs00961557_m1 for IR mRNA detection, all Applied Biosystems, Weiterstadt, Germany), 10 µl TaqMan® Fast Universal PCR Master Mix 2x (Applied Biosystems, Weiterstadt, Germany), 1 µl (300-900ng/µl) template and 8 µl H_2O (DEPC treated DI water, Sigma, Taufkirchen, Germany). Thermical cycling conditions were: 20 sec at 95°C, followed by 40 cycles of amplification with 3 sec at 95°C and 30 sec at 60°C. The ABI PRISM 7500 Fast (Applied Biosystems, Weiterstadt, Germany) was used to perform the PCR assays.

Quantification was carried out by the $\Delta\Delta$Ct-method using glyceraldehyde phosphate dehydrogenase (GAPDH) or beta-2-microglobulin as housekeeping genes (Hs99999905_m1 assay for GAPDH mRNA detection and Hs00984230_m1 for beta-2-microglobulin mRNA detection, both Applied Biosystems, Weiterstadt, Germany).

Double Immunofluorescence staining

For the characterisation of LR and IR expressing cells in placental tissue cryosections were examined. All samples were fixed in 5% buffered formalin. Antibodies used for the experiments are listed in Table 3. EVTs were examined with CK7 (Novocastra, Berlin, Germany and Santa Cruz Biotechnology, USA) as a specific marker and Prolactin (Dako, Glostrup, Denmark and AbD Serotec, Oxford, UK) was used as a specific marker for glandular epithelial cells of the decidua. CK7 (1:100), Prolactin (1:500) and IR (1:20) were diluted and incubated with the slides overnight at 4°C. CK7 and Prolactin were incubated with LR (1:2000), and IR with CK7 (1:30) and Prolactin (1:500) for 60 minutes at room temperature.

After washing, Cy2- and Cy3-labeled antibodies (all Dianova, Hamburg, Germany), served as a second antibody. For CK7 and Prolactin, we used goat-anti-mouse IgG Cy3 (1:500) and goat-anti-rabbit-IgG Cy3 (1:500), which will appear red, for Insulin receptor we used goat-anti-rabbit Cy2 (1:100) and for Leptin receptor goat-anti-mouse Cy2 (1:100) which will appear green. The slides were finally embedded in mounting buffer containing 4´,6-Diamino-2-phenylindole (DAPI) resulting in blue staining of the nucleus. Slides were examined with a Zeiss (Jena, Germany) Axiophot photomicroscope. Digital images were obtained with a digital-camera system (Axiocam, Zeiss, Jena, Germany) and saved on computer.

Results

Leptin receptor

We identified the expression of LR in the cytoplasm and apical cell membrane of villous trophoblasts (VT), extravillous trophoblasts (EVT) and glandular epithelial cells of the decidua in first trimester normal and abortive placentas as well as in hydatidiform mole.

Leptin receptor expression was up-regulated in the VT of hydatiform mole when compared to normal pregnancy ($p<0.001$)Figure 1a. With regard to abortion, the expression of LR in the VT was also increased, but without reaching significance (Figure 1a).

In abortive tissue ($p=0.007$) as well as in hydatidiform mole ($p<0.001$), LR expression in the EVT was significantly up-regulated in comparison to normal pregnancy (Figure 1b-d, 1e).

Both, mole pregnancy and abortion were accompanied by a significant up-regulation of LR ($p=0.001$; $p=0.005$) in glandular epithelial cells of the decidua as compared to normal pregnancy (Figure 1f).

Insulin receptor

Expression of IR was seen in the cytoplasm and apical membrane of VT, EVT and glandular epithelial cells of the decidua in first trimester normal and abortive placentas as well as in hydatidiform mole. Fetal blood vessels, decidual stromal cells and decidual maternal blood vessels were also examined. Insulin receptor expression in the VT was significantly increased in abortive tissue ($p=0.003$) and also in hydatidiform mole ($p=0.001$) compared to normal first trimester pregnancy (Figure 2a-d).

Both, mole pregnancy and abortion were accompanied by an increase of IR expression in EVT compared to normal pregnancy (both $p<0.001$), (Figure 2e).

Insulin receptor expression in glandular epithelial cells of the decidua was significantly upregulated in abortive tissue ($p<0.001$) as well as in hydatidiform mole ($p<0.001$) compared to normal first trimester pregnancy (Figures 2f).

With regard to IR expression in villi fetal blood vessels, we found an increased expression in both abortive tissue ($p=0.028$) and tissue of hydatidiform mole ($p<0.001$), (Figure 2g).

In abortive tissue ($p=0.003$) as well as in hydatidiform mole ($p=0.001$), IR expression in decidual stromal cells was significantly upregulated in comparison to normal pregnancy (Figure 2h).

Both, mole pregnancy and abortion were accompanied by an increase of IR expression in decidual maternal blood vessels compared to normal pregnancy ($p=0.007$, $p<0.001$), (Figure 2i).

RT-PCR

Leptin-R and Insulin-R mRNA expression were analyzed in abortive and normal control placental tissue by quantitative RT-PCR. Leptin-R mRNA expression was increased 4.8-fold for Leptin-R exon boundary 19-20 or 2.2-fold for Leptin-R exon boundary 6-7 respectively compared to normal controls ($p=0.003$). A Leptin-receptor overlapping transcript and Leptin-receptor overlapping transcript-1 showed 1.15-fold upregulation in abortive material. Insulin-R mRNA was 1.3 fold higher in abortive placental tissue compared to normal controls ($p=0.013$) (Figure 3).

Double Immunofluorescence staining

CK7 serves as a specific marker for EVT. Extravillous trophoblast cells were identified as LR and IR expressing cells after co-incubation with CK7 (Figure 4). We also examined glandular epithelial cells of the decidua, where Prolactin serves as a specific marker. These cells were also identified as LR and IR expressing cells (Figure 5).

Discussion

Within the present study, numerous differences in the expression of LR and IR in normal and disturbed pregnancy occurred. Highest expression of LR and IR was present in VT, EVT and glandular epithelial cells of the decidua of hydatidiform mole, which were significantly higher as compared to normal pregnancy. This was also true with regard to LR and IR expression in VT, EVT and in glandular epithelial cells of the decidua of abortive compared to normal first trimester placenta.

Preliminary investigations revealed a possible role of leptin in normal and disturbed human pregnancy (Toth *et al.*, 2008). Leptin expression was lowest in miscarriage and highest in mole pregnancies. In contrast to trophoblast tissue, expression of leptin in glandular epithelial cells of the decidua was increased in miscarriage. With regard to LR expression, again placentas of mole pregnancies and of miscarriages showed higher expression than placentas of normal pregnancies. This was also true for IR expression. Both, mole pregnancy and miscarriage share a failure in placental (and fetal) development. This is accompanied by incomplete vascularisation, endocrine dysfunction and hypoxia. A possible involvement of LR expression in RM was already indicated by Choi *et al*. (2003) showing increased expression of LR

genes in chorionic villi of placentas from RM patients as compared to normal pregnancy. They were also able to demonstrate LR expression by RT-PCR chorionic villi, however, they did not further localize LR expression. Within our study, detailed localization of LR expression was possible at mRNA and protein level showing expression of LR in VT, EVT and glandular epithelial cells. In addition, we performed quantitative RT-PCR on different LR overlapping transcripts and LR exon-boundaries. LR overlapping transcript gene share the first and second exons with the LR (Kurokawa et al. 2008). Largest differences in LR expression were obtained with primer and probes designed between exon boundaries 19-20 (4.8-fold upregulation of LR mRNA in abortive tissue). This assay detects the intact form of the LR. Only 2.2 fold upregulation of abortive LR mRNA was obtained with primer and probes designed between exon boundaries 6-7. This assay also detects short or truncated forms of the LR (Uotani et al. 2006; Gao et al. 2008). Marginal upregulation was found in abortive material with primer and probes for LR overlapping transcript genes. These genes share only the first and second exons with the LR. Therefore we may conclude that abortive trophoblast cells express the long form of LR mRNA 4.8-fold compared with normal control trophoblasts.

Our work confirmed earlier work of Li *et al*. (2004) as the authors also showed significantly higher expression of leptin and LR in partial and complete mole pregnancies by immunohistochemistry. Although our results obtained by immunohistochemistry show semi-quantitative results and therefore are limited in giving exact numbers, immunohistochemistry is able to show exact location of protein expression. We performed real-time RT-PCR which is able to quantify gene expression in different tissues and we were able to confirm our immunohistochemistry results at mRNA level.

While we focused on placental tissue from the first trimester of human pregnancy, Henson *et al*. (1998) investigated the expression of leptin and leptin receptor by reverse transcriptase-polymerase chain reaction in human placental villous tissue from term and earlier gestation (7-14 weeks). Leptin mRNA declined from earlier to late gestation suggesting an ontogenetic decline in leptin mRNA with advancing gestation.

Castellucci *et al*. (2000) were able to demonstrate a strong expression of the LR in the distal extravillous cytotrophoblastic cells of cell columns invading the basal plate, whereas leptin expression was homogeneously expressed in all the cellular components of cell columns. Leptin was able to increase the secretion of immunoreactive MMP-2 and fetal fibronectin in a dose-dependent manner and enhanced the activity of MMP-9 in cultured cytotrophoblastic cells. They concluded that leptin and LR could play a role in the invasive processes of the extravillous cytotrophoblastic cells by modulating the expression of MMPs. A possible role of IR in the invasion process was also demonstrated by Diaz *et al.* (2007) showing that the IR regulates choriocarcinoma cell invasion.

Within our study, LR and IR expression is up-regulated in both mole and abortive placental tissue. In hydatidiform mole pregnancy invasion of the trophoblast is disregulated. In abortive placentas the EVT is recognized by the maternal immune system and therefore invasion may also be disregulated. Therefore up-regulation of LR and IR in abortive EVT may be a compensation for this effect.

Identification of LR and IR expression in syncytiotrophoblasts, cells also responsible for the production of hormones vital to pregnancy maintenance, suggest a potential for autocrine or paracrine interactions within this tissue. The majority of IR and LR are expressed on structures that are currently assumed to drive placental

growth, i.e. syncytial sprouts and mesenchymal villi in first-trimester placentas and fetal endothelium at term. Therefore we hypothesize a growth promoting function, among others, of LR and IR in the placenta.

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Legends:

Figure 1

Fig. 1b Expression of Leptin receptor in normal human extravillous trophoblast cells, 10 x lens.

Fig. 1c Expression of Leptin receptor is significantly enhanced in abortive extravillous trophoblast cells (p=0.007), 10 x lens.

Fig. 1d Expression of Leptin receptor is significantly enhanced in hydatidiform mole extravillous trophoblast cells (p<0.001), 10 x lens.

Fig. 1a Summary of staining results (IRS-Scores) of immunohistochemical localisation of Leptin receptor in human villous trophoblast cells in normal first trimester pregnancy, abortion and hydatidiform mole (mean+SEM).

Fig. 1e Summary of staining results (IRS-Scores) of immunohistochemical localisation of Leptin receptor in human extravillous trophoblast cells in normal first trimester pregnancy, abortion and hydatidiform mole (mean+SEM).

Fig. 1f Summary of staining results (IRS-Scores) of immunohistochemical localisation ofLeptin receptor in human glandular epithelial cells of the decidua in normal first trimester pregnancy, abortion and hydatidiform mole (mean+SEM).

Figure 2

Fig. 2a Expression of Insulin receptor in normal human extravillous trophoblast cells, 25 x lens.

Fig. 2b Expression of Insulin receptor is significantly enhanced in abortive extravillous trophoblast cells (p=0.030), 25 x lens.

Fig. 2c Expression of Insulin receptor is significantly enhanced in hydatidiform mole extravillous trophoblast cells (p=0.001), 25 x lens.

Fig. 2d Summary of staining results (IRS-Scores) of immunohistochemical localisation of Insulin receptor in human villous trophoblast cells in normal first trimester pregnancy, abortion and hydatidiform mole (mean+SEM).

Fig. 2e Summary of staining results (IRS-Scores) of immunohistochemical localisation of Insulin receptor in human extravillous trophoblast cells in normal first trimester pregnancy, abortion and hydatidiform mole (mean+SEM).

Fig. 2f Summary of staining results (IRS-Scores) of immunohistochemical localisation of Insulin receptor in human glandular epithelial cells of the decidua in normal first trimester pregnancy, abortion and hydatidiform mole (mean+SEM).

Fig. 2g Summary of staining results (IRS-Scores) of immunohistochemical localisation of Insulin receptor in villi fetal blood vessels in normal first trimester pregnancy, abortion and hydatidiform mole (mean+SEM).

Fig. 2h Summary of staining results (IRS-Scores) of immunohistochemical localisation of Insulin receptor in decidual stromal cells in normal first trimester pregnancy, abortion and hydatidiform mole (mean+SEM).

Fig. 2i Summary of staining results (IRS-Scores) of immunohistochemical localisation of Insulin receptor in decidual maternal blood vessels in

normal first trimester pregnancy, abortion and hydatidiform mole (mean+SEM).

Figure 3

Fig. 3 Expression of Leptin receptor mRNA (exon boundaries 19-20 & 6-7), Leptin receptor overlapping transcript mRNA + Leptin receptor overlapping transcript 1 mRNA, and in addition Insulin receptor mRNA in abortive placental tissue compared to normal controls

Figures 4

Fig. 4a-c Leptin receptor (a) and CK7 (b) are expressed in EVT. Triple filter excitation shows expression of Leptin receptor and CK7 in the same type of cells (c), all pictures 20x lens.

Fig. 4d-f Insulin receptor (d) and CK7 (e) are expressed in EVT. Triple filter excitation shows expression of Insulin receptor and CK7 in the same type of cells (f), all pictures 20x lens.

Figures 5

Fig. 5a-c Leptin receptor (a) and Prolactin (b) are expressed in glandular epithelial cells of the decidua. Triple filter excitation shows expression of Leptin receptor and Prolactin in the same type of cells (c), all pictures 20x lens.

Fig. 5d-f Insulin receptor (d) and Prolactin (e) are expressed in glandular epithelial cells of the decidua. Triple filter excitation shows expression of Leptin receptor and Prolactin in the same type of cells (f), all pictures 20x lens.

Tables

characteristic	normal pregnancy n=14	abortion n=14	mole n=14	p value (Kruskal-Willis-Test)
maternal age (years)	27.6 ± 7.4 (16-43)	30.9 ± 6.0 (22-41)	31.0 ± 5.1 (23-40)	0.287
gestational age (weeks)	8.6 ± 1.8 (6-12)	8.9 ± 1.8 (6-12)	8.9 ± 1.7 (6-12)	0.993
gravidity	2.6 ± 1.5 (1-6)	2.8 ± 1.6 (1-7)	2.0 ± 1.1 (1-4)	0.351
parity	0.9 ± 1.0 (0-3)	0.6 ± 1.4 (0-5)	0.4 ± 0.5 (0-1)	0.277

Table 1. Demographic and clinical characteristics of study population. Values are given as mean±SD; the range is given in parentheses. P values not significant

	Number of slides		
gestational age	**control**	**abortion**	**mole**
6th week	2	1	1
7th week	1	3	2
8th week	3	2	3
9th week	3	3	3
10th week	2	2	2
11th week	2	2	2
12th week	1	1	1
	n=14	**n=14**	**n=14**

Table 2. Number of slides used for immunohistochemical staining for each week of gestational age

antibody	isotype	clone	dilution	source
Leptin receptor	Mouse IgG	MAB867	1:2000	R&D Systems, Minneapolis, USA
Insulin receptor	Rabbit IgG	AHP 1216	1:20	AbD Serotec, Oxford, UK
CK 7 for Insulin receptor	Mouse IgG	OV-TL 12/30	1 : 30	Novocastra, Berlin, Germany
CK 7 for Leptin receptor	Rabbit IgG	polyclonal	1 : 100	Santa Cruz Biotechnology, USA
Prolactin for Leptin receptor	Rabitt IgG	polyclonal	1 : 500	Dako, Glostrup, Denmark
Prolactin for Insulin receptor	Mouse IgG	MCA 712	1 : 500	AbD Serotec, Oxford, UK
goat-anti-mouse Cy3	Goat IgG	polyclonal	1:500	Dianova, Hamburg, Germany
goat-anti-rabbit Cy2	Goat IgG	polyclonal	1:100	Dianova, Hamburg, Germany
goat-anti-mouse Cy2	Goat IgG	polyclonal	1:100	Dianova, Hamburg, Germany
goat-anti-rabbit Cy3	Goat IgG	polyclonal	1:300	Dianova, Hamburg, Germany

Table 3. Antibodies used for immunohistochemical characterisation of decidual tissue samples